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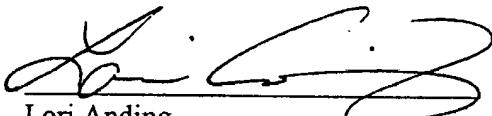
### **Japanese Patent:**

(11) Kokai (Unexamined Patent) Number: 57-185220

ANTI-CANCER DRUG HAVING CHLOROPHYLL  
DERIVATIVE EFFECTIVE COMPONENT

Corporate Translations Inc., hereby certifies that to the best of our knowledge and belief, has made an accurate and complete translation from Japanese to English of the original patent referenced above. The project has been adeptly managed through the three-phase quality process by three different experts: the translator, editor and proofreader. The translation team was specifically selected for their expertise in Medical/Research/Japanese/Patents to insure an accurate translation.

All necessary information including qualifications and expertise for the translation teams is on file at Corporate Translations Inc.



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(54) ANTI-CANCER DRUG HAVING CHLOROPHYLL DERIVATIVE EFFECTIVE COMPONENT

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Specifications

1. Title of the Invention: Anticancer Drug Having Chlorophyll Derivative Effective Component

## 2. Scope of the Patent=s Claims

An anticancer drug having as an effective component a chlorophyll derivative, disclosed by the general formula:

general formula

[insert formula]

(in this formula, X indicates an H atom or OH base, Y indicates a  $\text{-COOCH}_3$  base or H atom, Z is a Mg atom or 2 H atoms (position 13, 14).

## 3. Detailed Explanation of the Invention

This invention relates to a novel type of an anticancer drug having as an effective component a chlorophyll derivative.

More specifically, this invention relates to an anticancer drug having as an effective component a chlorophyll derivative, disclosed by the general formula:

general formula

[insert formula]

(in this formula, X is an H atom or OH base, Y indicates a  $\text{COOCH}_3$  base or H atom, Z is a Mg atom or 2 H atoms (position 13, 14).

Chlorophyll derivatives expressed by the formula above have not been known at all from prior art.

[page 2]

The inventors of this invention have already discovered 10-hydroxy phaeophorbide (hereinafter referred to as OH-Phd) which displays an extremely powerful optical activity, developed from Chlorella cells treated according to a specific treatment (Nichidoka, Summary of Lectures from the 55<sup>th</sup> Conference, see pages 476, 477). Next, when they were developing the physiological effect of the product, at the point when a chlorophyll derivative disclosed by the formula above was added, the inventors discovered that this not only caused selective cumulation in tumor cells via normal cells, but also that the discharge from the tumor cells was slow, and that the growth of tumors was suppressed. This was observed by the authors during irradiation conducted with visible rays in the range of 400 ~ 700 nm. The authors also discovered that tumor

cells were destroyed, that discharge from normal organs and cells occurred quickly, that absolutely no reaction occurred in a dark environment and there was no toxicity.

The present invention is based on this discovery.

The following compounds can be used for the chlorophyll derivative expressed by the formula above.

Name	1) Symbols in the Formula			2) Abbreviation
	X	Y	Z	
10-hydroxyphaeophorbide <u>a</u>	-OH	-COOCH <sub>3</sub>	2H	OH-Phd
phaeophorbide <u>a</u>	-H	-COOCH <sub>3</sub>	2H	Phd
pyrophaeophorbide <u>a</u>	-H	-H	2H	Pyrophd
10-hydroxychlorophyllide <u>a</u>	-OH	-COOCH <sub>3</sub>	Mg	OH-Chld
chlorophyllide <u>a</u>	-H	-COOCH <sub>3</sub>	Mg	Chld
pyrochlorophyllide <u>a</u>	-H	-H	Mg	Pyrochld

Notes:

- 1) X indicates position 10, Y indicates position 11 in the orientation of each substance.  
-2H of Z indicates position 13 and position 14 in the orientation, Mg is linked to each N.

## 2. Materials Used in the Specifications

In recent years, it was disclosed (by T.J. Dougherty et al., in Cancer Research, 38, 2628 ~ 2635, 1978) that the optical effect of hematoporphyrin derivatives was tested for the purposes of treatment of tumors. With respect to said phaeophorbides, it was discovered that OH-Phd is characterized by a relatively high optical activity when compared to hematoporphyrin, as well as by a high selective cumulation in tumors and a quick discharge from normal organ cells.

This substance is activated in particular in the optical wavelength band in the range of 400 ~ 700 nm, it has a high transmissivity in living organism in the wavelength band range of 600 ~ 700 nm (the effective wavelength region is 640 ~ 690 nm), and the optical activity of this substance (activity per unit of time, per irradiating energy, and the dissolving amount of biological components per unit of administration) is as much as 10 times higher than that of hematoporphyrin (effective wavelength is 630 nm).

Phaeophorbides are non-toxic in living organisms in a dark environment, and even with visible light rays in the range of 400 ~ 700 nm, if the light rays per se are not toxic.

Accordingly, this makes it possible to destroy tumor cells very effectively and also very safely through irradiation after controlled administration.

Glass fibers developed in recent years made it possible to perform irradiation with light rays in the internal parts of bodily organs. In addition, it was also confirmed that infrared light in the wavelength range of 600 ~ 700 nm can be used for effective transmission of energy inside living organism up to about 3 cm. This means that basically all regions in tumors can be reached with irradiating light rays.

Furthermore, because very narrow directivity can be achieved by using laser light rays which have excellent condensing characteristics, this makes it possible to increase the effect of the reaction.

The optical effect is essentially caused by an excitation of a photosensitized substance contained in a living organism due to the energy of visible light rays. Subsequently, a safe oxygen activation occurs when active oxygen is generated (a certain type of oxygen,  $^1\text{O}_2$ ). This is followed by oxidation and decomposition of lipids present in a living organism, as well as of protein nucleic acids, etc. Because the resulting effect is destruction to cells, this can cause indiscriminate damage to cells when a living organism that includes a photosensitized substance is irradiated by light rays. However, as long as this photosensitized substance is cumulated selectively in tumor cells, the tumor cells can be destroyed without exerting an influence on normal cells.

The following is a detailed explanation of this invention.

The method that was used to manufacture chlorophyll derivatives utilized for the anticancer drug of this invention will be explained first.

Said chlorophyll derivative manufacturing method is characterized by the fact that chlorophyllase contained in cells of green plants containing chlorophyll is utilized, as well as oxidation with dephytylization achieved by oxidation oxygen.

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This method is thus a chemical manufacturing method using plants as inactivated raw material together with oxidation oxygen and chlorophyllase in cells or with isolated chlorophyll.

Among the raw materials that can be used with a method characterized by conducting oxidation and dephytylation with oxidation oxygen via chlorophyllase in cells of plants or chlorophyll in green plants are plants containing chlorophyll and having chlorophyllase activity and oxidation oxygen activity. Although any such plants can be utilized, it is best to use plants characterized by a large content of chlorophyll and a high level of oxygen activity for the purposes of mass production on an industrial scale. For instance Chlorella, Senedesmas, and similar green algae can be used as raw material from the viewpoint of the yield, the economic characteristics, etc.

The following is a detailed explanation of a concrete example of a method to manufacture said chlorophyll derivative by using Chlorella as the raw material.

After 10-hydroxychlorophyllide a was derived with oxidation oxygen from Chlorella cells containing chlorophyll a, Chlorella cells were cultivated again with a common method using derivation of 10-hydroxychlorophyllide via chlorophyllase inside the cells and by removing the carbon source, or in a buffer solution such as a phosphate buffer solution (pH 7.0), preferably at a temperature that is 5EC higher than a suitable temperature for Chlorella cells (about 40EC). The processing was conducted for a period of 6 ~ 48 hours while the culture was stirred with an air current. (Treatment Solution - A).

After an organic solvent was then added to the treatment Solution - A obtained in this manner, for example acetone, methanol, ethanol (with a concentration of up to 70%, although an optimal concentration is 30%), the solution was aged for a period of 3 hours, preferably when the optimal temperature was reached (36EC), at a temperature conducive to the chlorophyllase effect in chlorophyll. (Aged Solution - B).

These operations were used to form an oxidized OH base containing hydrogen in position 10 in chlorophyll. After the phytol base was substituted by H from position 12 in the chlorophyllase, this made it possible to obtain chlorophyllide a with phytol in position 12 forming H without oxidation of 10-hydroxychlorophyllide a and position 10 in chlorophyll.

Chlorophyll pigment extraction can be used with generated OH-Chld and Chld according to a common method and a common refining method can then be used for isolation. For example, after centrifugal separation is applied to Aged Solution B, the supernatant is formed, methanol is then added again to the residue and the pigment is extracted. The supernatant is mixed with the extract and after the mixed solution is enriched under reduced pressure, chloroform is added and mixing is applied again. After that, distilled water is added which is followed by washing. The chloroform layer is removed so that a residue dissolved in ethanol can be obtained after chloroform is removed under reduced pressure. Then, separation can be conducted with thin layer chromatography, etc., and through distribution using a 17% hydrochloric acid solution, which makes it possible to obtain OH-Chld and Chld.

In addition, while the above described OH-Chld and Chld manufacturing method was used to obtain Treatment Solution - A used for Chlorella processing, and Aged Solution - B was obtained after that, a heat treatment was applied for 30 minutes at 70EC (50 ~ 80EC) to cells produced by Chlorella without preparing Treatment Solution - 1. Also, after suspension in acetone or another polar solvent with the above mentioned concentration, the product is allowed to age for 30 minutes to 3 hours at a temperature of 20 ~ 50 EC in a neutral pH and the phytol base in position 12 in chlorophyll is substituted by H from chlorophyllase, which makes it possible to obtain an optimal yield of chlorophyllide a. In addition, stirring can be applied with air current treatment for a period of about 8 ~ 24 hours at a temperature of 20 ~ 50 EC in a neutral pH in the acetone suspension solution with live Chlorella cells at the point when an optimal OH-Chld yield was obtained. Although the method to manufacture 10-hydroxychlorophyllide a or phaeophorbide a utilized OH-Chld or Chld as the raw material during the above described manufacturing process, it is also possible to obtain the same result with a method replacing a Mg atom in porphyrine ring with a hydrogen atom according to a commonly used method, for instance with hydrochloric acid processing.

Mg can be easily substituted with a H atom with the hydrochloric acid solution with common processing involving separation and refining of OH-Chld or Chld, making it possible to obtain OH-Phd or Phd.

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The OH-Phd or Phd obtained according to this invention can be mixed together or each item can be used as is. It is also possible to conduct separation and refining with thin film chromatography, etc. Pyrophaeophorbide a can be manufactured according to the method described by P.C. Pennington et al [J. Am. Chem. Soc., 86, 1418, (1964)].

Pyrophd can be obtained for instance by treating chlorophyll a with pyridine and treating the pyrochlorophyll obtained in this manner with hydrochloric acid, removing phytol base in position 12 and replacing it with a hydrogen atom and creating a hydrogen atom from a Mg atom in porphyrine ring. In case of a chemical manufacturing method using as raw material chlorophyll that was already isolated from a plant or that does not have oxidation oxygen characteristics or chlorophyllase activity in the cells, the target compound can be obtained with the same manufacturing method also when the plant displays oxidation oxygen activity and chlorophyllase activity is used as described above, with the exception of chemical oxidation and dephytylization.

In this case, after the derivation of the hydroxychlorophyll obtained from chlorophyll by a weak oxidation, OH-Chld can be separated with saccharose column chromatography and after that, dephytylization is applied through a treatment using 30% hydrochloric acid, enabling to obtain OH-Phd with a high yield.

The inventors of this invention were thus able to obtain highly active OH-Phd and OH-

Phd characterized by a low activity. The data obtained is shown below in Table 1.

Table 1

	High-Activity OH-Phd	Low-Activity OH-Phd
Molecular Formula	$C_{35}H_{36}O_6N_4$	$C_{35}H_{36}O_6N_4$
$E_{667}/E_{409}$	1.93	1.99
$R_f$ (TLC)	0.34	0.21
Chemical Shift (NMR)	8 4.73 7 4.47	4.45 4.09

Notes:

$E_{667}/E_{409}$ : The ratio between the maximum absorption of red color and the maximum absorption of blue color in absorption spectrum of visible parts.

$R_f$  (TLC): The  $R_f$  value obtained with a thin silica gel layer, 20 x 20 cm, 0.25 mm, development solvent, benzene, ethyl acetone, ethanol, n-propanol (14 : 4 : 1).

Chemical Shift 8 and 7:

The chemical shift in the proton in position 7 and 8 obtained with nuclear magnetic resonance.

As one can see from Table 1, the high-activity OH-Phd and low-activity OH-Phd can be considered an optical isomer configuration in hydrogen position 7, 8 with high-activity OH-Phd and low-activity OH-Phd.

The effective amount for administration of the chlorophyll derivative in the anticancer drug of this invention is in each case in the range of 10 mg ~ 300 mg per daily dose for an adult, while the range of 50 ~ 150 mg is preferred.

With respect to the formulation of the anticancer drug of this invention, the preparation can be administered orally or as a preparation that can be injected according to a customary method. If the preparation is injected, it can be used after it has been dissolved in distilled water because Phd and OH-Phd can be dissolved directly in a symbiotic physiological salt solution. In addition, Phd can also be neutralized after it has been first dissolved in a weak alkaline solution and then it can be mixed with a physiological salt solution.

Furthermore, it should be added that the anti-cancerous effect of the above-described substance is not limited only to the above described example of this invention or to a manufacturing example of the anti-cancer drug of this invention or a drug example which was tested for toxicity.

### Embodiment 1

$1.25 \times 10^6$  items of sarcoma 180 tumor cells were administered by subcutaneous inoculation to ICR mice (male mice, 7 weeks old, weighing about 25 g) per 1 mouse, and standard rearing was conducted after the grafting. The grafted group of individual mice in which growth of tumor cells was confirmed (10 mice in 1 group) was injected from the 8<sup>th</sup> day after the grafting with 0, 10, or 20 mg of OH-Phd per kilogram of body weight of the mice, dissolved in physiological salt solution, or with 20 mg/kg of Phd, Pyrophd, OH-Chld, Chld, or Pyrochld per kilogram of body weight of the mice, administered directly into the tumor region in the mice. This administration was conducted 9 times during a period of 3 days. Immediately after that, irradiation was conducted for 1 day, and for 6 hours, with rays in the wavelength range of 400 ~ 1,000 nm having a strength of 100 mW/cm<sup>2</sup> (with the light of a 500 W Tungsten lamp transmitted through 2 cm of a water layer providing a shielding profile for hot rays). The tumor was then extracted on the 32<sup>nd</sup> day after the grafting with the tumor took place, the weight of the tumor was measured and the tumor suppressing ratio was calculated based on the formula below.

$$\text{suppression ratio \%} = \frac{\text{average tumor weight in contrast segment} - \text{average tumor weight in tested segment}}{\text{average tumor weight in contrast segment}} \times 100$$

The contrast group of the mice was administered a physiological salt solution in the same manner as the tested segment and they were also irradiated in the tumor region. In addition, the mice were fed OH-Phd in a dark environment with 20 mg/kg of body weight, and also the contrast group was kept in a dark environment. The results are shown in Table 1.

[page 5]

Because Chld, OH-Chld, Pyrochld are unstable properties, a Mg atom can be easily released into a living organism or into the molecules used during extracting operations, causing changes in Phd, OH-Phd, and Pyrophd.

The anti-tumor activity of these substances, corresponding to Mg-Phd, is almost identical.

Table 1 - Result of the anti-tumor effect after direct administration into the tumor of the chlorophyll derivative.

	Administration Amount (mg/kg)	Light	Average Tumor Weight (g)	Suppression Ratio (%)
Contrast Segment	0	L	1.49 $\pm$ 1.46	0
OH-phd	90	L	0.35 $\pm$ 0.41	76.5
	180	L	0.19 $\pm$ 0.14	87.2
	180	L	0.75 $\pm$ 1.04	49.7
	180	D	1.43 $\pm$ 0.86	4.0
OH-Chld	180	L	0.23 $\pm$ 0.38	84.6
Phd	180	L	0.63 $\pm$ 0.32	57.7
Chld	180	L	0.54 $\pm$ 0.47	63.8
Pyrophd	180	L	0.86 $\pm$ 0.72	42.3
Pyrochld	180	L	0.57 $\pm$ 0.42	61.7

Notes:

L: Light rays with 20 Klux

L': Light rays with 0.5 Klux

D: Dark environment.

## Embodiment 2

Similarly to Embodiment 1, sarcoma 180 tumor cells were administered by subcutaneous inoculation into the dorsal region of ICR mice per 1 mouse and standard rearing was conducted after grafting. The grafted group of individual mice in which growth of tumor cells was confirmed was injected from the 8<sup>th</sup> day after the grafting with 0 mg, 0.3 mg, 1 mg, and 3 mg of OH-Phd and Phd per kilogram of body weight of the mice, dissolved in physiological salt solution, administered through the tail vein of the mice, resulting in a total of 11 administrations during an interval of 2 ~ 3 days. Similarly to Embodiment 1, irradiation with light rays was conducted and after a period of rearing lasting 32 days, the tumor was extracted and the tumor suppression ratio was determined.

Table 2 - Anti-Tumor Effect with Administration of OH-Phd into tail vein

	Total Administered Amount - mg/kg of Weight	Light Rays	Average Tumor Weight (g)	Suppression Ratio (%)
Contrast Group Segment	0	L	5.54 $\pm$ 1.04	0
Admin. Segment OH-Phd	3.3	L	1.05 $\pm$ 0.6	81.0
	11	L	0.68 $\pm$ 0.60	87.7
	33	L	0.18 $\pm$ 0.18	96.8
	33	L	2.36 $\pm$ 1.44	57.4
	33	D	4.43 $\pm$ 0.87	20.0
Admin. Segment Phd	3.3	L	1.6 $\pm$ 0.87	71.1
	11	L	1.3 $\pm$ 1.44	76.3
	33	L	2.3 $\pm$ 1.29	57.9

Notes:

L: 20 Klux

L\*: 0.5 Klux.

D: Dark environment.

An excellent effect was displayed with a joint administration of OH-Phd, Phd into the tail vein. The tumor was dissipated in about half of the mice in the group to which 33 mg of OH-Phd was administered.

### Embodiment 3

Sarcoma 180 tumor cells were administered by subcutaneous inoculation into the dorsal region of ICR mice and after 23 days of standard rearing, when the grafted tumor grew to an approximate size range of  $200 \sim 300 \text{ mm}^2$ , 3 mg/kg of the body weight of the mice was administered through the tail vein. After 24 hours, irradiation was applied with light having an optical strength of  $300\text{mw/cm}^2$  with shielded hot rays (using a 500 W light source, a Tungsten lamp) for 30 minutes to the tumor region. The administration of OH-Phd and treatment with irradiating light rays was repeated 3 times in daily intervals. After that, rearing was continued in a dark environment and the changes in the size of the tumor were observed. The segment which was not administered OH-Phd was given the same amount of physiological salt solution, while the light irradiation treatment was applied to this segment in the same manner.

Table 3 - Tumor treatment effect of OH-Phd

	Total OH-Phd Admin. Amount (mg/kg of weight)	Size of Tumor during Administration (mm <sup>2</sup> )	Tumor Size after 10 Days of Treatment (mm <sup>2</sup> )	Suppression Ratio %
Contrast Segment	0	273 $\pm$ 64	376 $\pm$ 123	0
Tested Segment	9	248 $\pm$ 82	129 $\pm$ 79	.66.0

Note:

The size of the tumor (mm<sup>2</sup>); long diameter x short diameter; after OH-Phd was administered. Because degenerative destruction occurred in the segment to which irradiation with light rays was applied, after 10 days, the size of the tumor has shrunk to about 1/3 (cubical conversion).

#### Embodiment 4

Similarly to Embodiment 1, sarcoma 180 was grafted to ICR mice and from the 8<sup>th</sup> day after the grafting, 0.10 mg OH-Phd was administered per kg of body weight of the mice orally by using a stomach probe. After the administration, the tumor region was irradiated with light rays for 30 minutes in 24 hour intervals in the same manner as in Embodiment 3 by using light rays with 300 mW/cm<sup>2</sup>. The administration and treatment with irradiating light rays was conducted twice during a continuous period of 5 days for a total of 10 administrations. After rearing was conducted for 32 days, the tumor was extracted, its weight was measured and the suppression ratio was calculated.

Table 4 - Anti-Tumor effect with oral administration of OH-Phd

	Total Administered Amount (mg/kg)	Average Tumor Weight (g)	Suppression Ratio (%)
Contrast Segment	0	2.6 $\pm$ 1.5	0.
OH-Phd Segment	100	0.9 $\pm$ 0.5	65.4

#### Embodiment 5 (Toxicity Test)

[page 6]

[part of the top line illegible]

... ICR mice (male and female) whose weight was around 50 [illegible] g were used to conduct acute toxicity tests for each administration path.

For oral administration, the substance was administered with a stomach probe after it was dissolved in distilled water. For intravenous administration and administration into the abdominal cavity, the substance was dissolved in a physiological salt solution and then injected with a syringe. LD<sub>50</sub> was calculated according to the Richfield-Wilcoxon method. Rearing was conducted after the administration in each case in a dark environment.

Table 5 - Acute toxicity characteristics in a dark environment (LD<sub>50</sub>) mg/kg

Administration Method	OH-Phd	Phd	Pyrophd
Intravenous Administration	200 <	200 <	200 <
Administration Into Abdominal Cavity	200 <	200 <	200 <
Oral Administration	1000 <	1000 <	1000 <

The numbers shown in the figure indicate the limit for solubility of the administered substance in water or in a physiological salt solution. Death did not occur in either case.

Although absorption and discharge of the substance into normal cells and into organs, as well as hypersensitivity to light rays, was displayed with irradiation with light rays within 12 hours after the administration, no reaction was indicated with irradiation 24 hours after the administration. The administered substance was not observed in any cells or organs 24 hours after the administration.

#### Manufacturing Example 1

After Chlorella cells (1 kg of moist substance) were suspended in 5 l of a phosphate buffer solution (0.1 M, pH 7.0) and stirring was conducted for a treatment period of 48 hours with an air current at 40EC, the Chlorella cells were gathered by centrifugal separation and these cells were then dissolved in 3 l of a 30% acetone solution and then allowed to age for 3 hours at 36EC.

After the aging, centrifugal separation was conducted, the supernatant was extracted and the extract solution was obtained after 3 l of methanol were added 3 times to the residue.

The supernatant obtained in this manner mixed with the methanol extract solution was

then enriched under reduced pressure to 2 of the amount and after 2 l of chloroform were added to the resulting mixture, vigorous mixing was applied. Distilled water was added after that and washing was conducted, which was followed by separation conducted with a chloroform layer, making it possible to obtain a residue when the chloroform was removed under reduced pressure. The residue obtained in this manner was dissolved in 1 l of ethyl ether, and after an equivalent amount of 17% HCl solution was added, separation was conducted with a layer of hydrochloric acid solution. After the concentration of the hydrochloric acid was diluted to 5% with water, ethylene ether was added and the solution was mixed.

After this ether layer was separated and washing with pure water was conducted, the resulting mixture was enriched under reduced pressure. This enriched solution was applied to a silica gel layer and the coloring band ( $R_f$  0.39 and  $R_f$  0.34) was developed with a solvent (benzene, ethyl acetone, ethanol, n-propanol, 16 : 4 : 1 : 1) in a cool and a dark environment.

The coloring band obtained in this manner was then scraped off, methanol was added and the pigment was extracted, and after evaporation under reduced pressure in methanol, pigments (Phd 1.19 g, OH-Phd 0.86 g) were obtained.

#### Manufacturing Example 2

Acetone with a concentration of 30% was added to an enriched solution of live Chlorella cells, and after strong ventilation was conducted with a pH of 7.0 and a temperature of 36EC for a period of 24 hours, the chlorophyll pigment was manufactured with extraction, separation, and refining according to the same manner as in Embodiment 1.

492 mg of Phd and 386 mg of OH-Phd were obtained from 100 g of Chlorella algae.

#### Manufacturing Example 3

After dried Chlorella powder (inactivated chlorophyllase) was pulverized with a homogenizer to obtain a fine powder, the powder was suspended in a 30% acetone solution, and after ventilation and stirring was conducted with an air current for a period of 24 hours, a chlorophyll-based pigment was obtained according to the same method that was used in Manufacturing Example 1. Next, a hydroxy chlorophyll fraction was separated with saccharose column chromatography (0.5% development solvent comprising isopropanol - petroleum ether), and after the solvent was distilled out, the resulting mixture was dissolved in ethanol ether. Next, an equivalent amount of 30% hydrochloric acid was added and after vibrations were applied in a dark environment at room temperature for a period of 1 hour and after dephytylization was conducted, water was added, as well as a 17% hydrochloric acid concentration. Ether was then added to achieve pigment distribution, a 17% HCl fraction was formed and refining was conducted in the same manner as in the case of Manufacturing Example 1. This made it possible to obtain 618 mg of OH-Phd from 100 g of Chlorella algae.

#### Manufacturing Example 4

100 mg of chlorophyll a was dissolved in 50 ml of acetone and 60 g of silica gel (soda silicate) was added for adsorption of chlorophyll, and after acetone was evaporated, aging was conducted for 1 hour at 36EC in a dark environment with air current ventilation. After the adsorption, the adsorbed pigment was dissolved in acetone and enriched under reduced pressure at a low temperature in a dark environment.

[page 7]

After dissolution in ethylene ether, the hydroxy chlorophyll fraction was separated with saccharose column chromatography in the same manner as in Embodiment 3, the hydroxy chlorophyll fraction was scrapped off, dissolved in ether, an equivalent amount of a 30% HCl solution was added, and after dephytylization, water was added to create a 17% concentration of hydrochloric acid. This was followed by the same refining process that was also used in Manufacturing Example 1.

This made it possible to obtain 32 mg of OH-Phd from 100 mg of Chlorella.

#### Preparation Manufacturing Example 1

After 15 mg of OH-Phd was dissolved in 0.5 ml of sterilized distilled water and after dilution was conducted with 0.5 ml of 1.8% physiological salt solution, the mixture was filtered through a bacteria removing filter, poured into an ampule for sterile injection and stored in a dark environment.

#### Preparation Manufacturing Example 1

After 1,500 mg of a mixture consisting of 630 mg of OH-Phd and 870 mg of Phd was dissolved in 50 ml of 0.1 N NaOH, approximately 50 ml of 0.1 N HCl was added to achieve neutralization. In addition, the mixture was filtered through a bacteria removing filter, poured into an ampule for sterile injection, sealed and stored in a dark environment.

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⑮ クロロフィル誘導体を有効成分とする制ガン

剤

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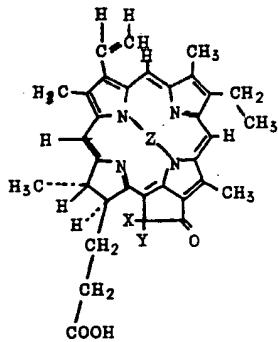
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明 細 書

1. 発明の名称 クロロフィル誘導体を有効成分とする制ガン剤

2. 特許請求の範囲

一般式

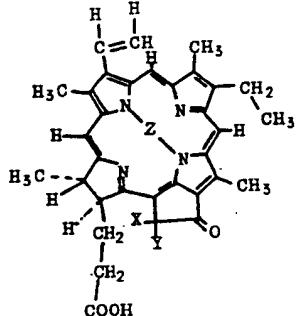


(式中 X は H 原子又は OH 基であり、 Y は -COOCH<sub>3</sub> 基又は H 原子であり、 Z は Mg 原子又は 2 ケの H 原子 (13, 14 位) を表わす) で示されるクロロフィル誘導体を有効成分とする制ガン剤。

3. 発明の詳細な説明

本発明はクロロフィル誘導体を有効成分とする新規な制ガン剤に関する。

さらに詳しく言えば、本発明は、一般式



(式中 X は H 原子又は OH 基であり、 Y は -COOCH<sub>3</sub> 基又は H 原子であり、 Z は Mg 原子又は 2 ケの H 原子 (13, 14 位) を表わす) で示されるクロロフィル誘導体を有効成分とする制ガン剤に関する。

上記式で表わされるクロロフィル誘導体が制ガン剤として使用し得ることについては従来、全く知られていない。

本発明者らは、先にクロロフィルを多量に含むクロレラの特殊処理細胞から極めて強力な光力学的活性を示す 10-ヒドロオキシフェオ

フォルバイド<sub>a</sub>（以下OH-Phdと記す）を見出した（日農化、昭和55年度大会講演要旨集476,477参照）。次いでその生理的作用操作について研究していたところ、前記式で表わされるクロロフィル誘導体を動物に投与するときは、これが、正常細胞より、腫瘍細胞に選択的に蓄積し、且つ腫瘍細胞からの排泄がおそいこと、可視光線400～700nmの光を照射したとき腫瘍に腫瘍の増殖を抑制し、腫瘍細胞を破壊すること、正常な臓器、細胞からは速やかに排泄されること、暗所では全く反応せず無害であることを見出した。

本発明はかかる知見に基づくものである。  
前記式で表わされるクロロフィル誘導体としては、下記の化合物をあげることができる。

からの排泄が速いことが見出された。特にこの物質を活性化する光波長域（400～700nm）中、生体への透過性の高い600～700nm（有効波長640～690nm）の波長域におけるこの物質の光力学的活性（単位時間当たり、照射光エネルギー当たり、単位投与量当たりの生体成分の分解量）はヘマトポルフィリン（有効波長630nm）のそれより10倍も高い。

フェオフォルバイド類は生体に対し、暗所では無害であり、又、可視光線400～700nmもまた、それ自体は無害な光線である。

従つて、管理されたこれらの投与の後の光照射により極めて安全に且つ強力に腫瘍細胞を破壊することができる。

近年グラスファイバーが発達し臓器内部迄光照射が可能となつており、また、波長600～700nmの赤色光は生体組織内部約3cm迄有効強度のエネルギーが透過することが、確認されており、これらのことから殆んどの部位の腫瘍へ光照射が可能となるものと解される。さらに、

名 称	1) 式中の記号			2) 略名
	X	Y	Z	
10-ヒドロオキシフェオフォルバイド <sub>a</sub>	-OH	-COOCH <sub>3</sub>	2H	OH-Phd
フェオフォルバイド <sub>a</sub>	-H	-COOCH <sub>3</sub>	2H	Phd
ピロフェオフォルバイド <sub>a</sub>	-H	-H	2H	Pyrophd
10-ヒドロオキシクロロフライド <sub>a</sub>	-OH	-COOCH <sub>3</sub>	Mg	OH-Chld
クロロフライド <sub>a</sub>	-H	-COOCH <sub>3</sub>	Mg	Chld
ピロクロロフライド <sub>a</sub>	-H	-H	Mg	Pyrochld

註)1. Xは10位、Yは11位に各々配位する。

Zの-2Hは各々13, 14位に配位、Mgは各Nと配位結合

## 2. 本明細書中において使用

近年、ダハティらは（T.J.Dougherty et al. Cancer Research 38, 2628～2635 1978）ヘマトポルフィリン誘導体を用いてそれらの光力学的作用を利用し、腫瘍の治療を試みている。上記のフェオフォルバイド類、就中OH-Phdはこのヘマトポルフィリンに比較し光力学的活性が高く、腫瘍への選択的蓄積性<sup>1</sup>大きく、正常な臓器細胞

光源として鋭い指向性をもち、集光性のすぐれたレーザー光線を用いれば、より反応を高めることができる。

光力学的作用は、本来、生体に取り込まれた光増感物質が可視光線のエネルギーによつて励起され、次いで安定な酸素を活性化して、活性酸素（一重項酸素<sup>1</sup>O<sub>2</sub>）を生成し、これが生体成分中の脂質、蛋白質、核酸等の酸化分解し、細胞の破壊をもたらす作用であるので、光増感物質を取りこんだ生体に光が照射されれば、無差別に細胞の損傷が生ずるのであるが、その光増感物質が腫瘍細胞に選択的に蓄積する物質であれば正常細胞に影響を及ぼすことなしに腫瘍を破壊せしめることができる。

以下に本発明をさらに詳細に説明する。

はじめに、本発明の制ガン剤に用いるクロロフィル誘導体の製造方法について記す。

上記のクロロフィル誘導体の製造方法には、緑色植物中のクロロフィルを植物の細胞内のクロロフィラーゼ、及び酸化酵素で酵素的に脱フ

イチール化し、酸化することを特徴とする方法とすでに単離されているクロロフィルあるいは細胞内のクロロフィラーゼや酸化酵素の不活化された植物を原料として化学的に製造する方法とがある。

緑色植物中のクロロフィルを植物の細胞内のクロロフィラーゼ、及び酸化酵素で酵素的に脱イチール化及び酸化を行うことを特徴とする方法で用いる原料としては、クロロフィルを含みかつクロロフィラーゼ活性及び酸化酵素活性のある植物は、任意に利用することができるが、クロロフィル含量が多く酵素活性が高く、かつ、工業的に大量生産が可能である植物、例えばクロレラ、セネデスマスの様な緑色微細藻類等を原料とするのが、収率、経済性等の点からみて有利である。

以下、クロレラを原料とした場合の上記のクロロフィル誘導体の製造法の具体例についてさらに詳細に説明する。

クロレラ細胞中のクロロフィル $\alpha$ を細胞内酸

12位のフィチール基がHとなつたクロロフィライド $\alpha$ を得ることができる。

生成したOH-Chl $\alpha$ 及びChl $\alpha$ は通常行なわれているクロロフィル類色素の抽出、精製方法に従い単離することができる。例えば、静置液-Bを遠心分離した後、上清をとり、残渣は更にメタノールを加えて色素を抽出し、上清、抽出液の混液を減圧下濃縮後クロロホルムを加えて混合した後、さらに蒸留水を加えて水洗を行ないその後クロロホルム層をとり減圧下クロロホルムを留去して得られる残渣をエーテルに溶解した後、17%塩酸溶液との液々分配更に薄層クロマトグラフィ等により分離することにより、OH-Chl $\alpha$ 及びChl $\alpha$ を得ることができる。

また、前述のOH-Chl $\alpha$ 及びChl $\alpha$ の製造法において、クロレラを処理して処理液-Aとした後に静置液-Bを得たのであるが、処理液-Aとすることなしにクロレラ生細胞を70℃(50~80℃)30分加熱処理、又はアセトン等の極性溶媒に前記濃度に懸濁し、pH中性附近、温

化酵素で10-ハイドロオキシクロロフィル $\alpha$ に誘導し更に細胞内クロロフィラーゼにより10-ハイドロオキシクロロフィライドを誘導する目的でクロレラを培養する際に通常用いられている培地より炭素源を除いた培地、あるいはリン酸緩衝液(pH7.0)のような緩衝液中で好ましくはクロレラ細胞の適温より約5℃高い温度(約40℃)で、通気搅拌を行ないながら6~48時間処理する。(処理液-A)

得られた処理液-Aに水溶性の有機溶媒、例えばアセトン、メタノール、エタノール(70%までの濃度、最適濃度30%)を加えてクロロフィル中のクロロフィラーゼの作用温度内、好ましくは至適温度(36℃)にておよそ3時間静置する。(静置液-B)

これらの操作によりクロロフィル中の10位の水素が酸化されてOH基となり、クロロフィラーゼにより12位のフィチール基がHに置換された10-ハイドロオキシクロロフィライド $\alpha$ 及びクロロフィルの10位が酸化されておらず、

度20~50℃で30分ないし3時間静置することによりクロロフィル中の12位のフィチール基をクロロフィラーゼによりHに置換することにより、クロロフィライド $\alpha$ をより良好な収率で得ることができる。またOH-Chl $\alpha$ を良好な収率で得たい時にはクロレラ生細胞のアセトン懸濁液をpH中性附近、温度20~50℃、通気搅拌処理を約8~24時間行うことによつても得られる。10-ハイドロオキシフェオフォルバイド $\alpha$ あるいはフェオフォルバイド $\alpha$ の製造方法は、上述のごとくして製造したOH-Chl $\alpha$ あるいはChl $\alpha$ を原料として、通常行なわれるポルフィリン環のMg原子を水素原子に置換する方法、例えば塩酸で処理する方法に従い、それぞれ得ることができる。

通常OH-Chl $\alpha$ あるいはChl $\alpha$ を分離精製する工程で用いる塩酸溶液により容易にMgがH原子に置換し、OH-PhdあるいはPhdとして得られる。

本発明においては、上記で得られたOH-Chl $\alpha$ とChl $\alpha$ 、あるいはOH-PhdとPhdは夫々混合物の

まま使用することもできるがこれらは必要に応じ薄層クロマトグラフィー等で分離精製してもよい。ピロクロロフィライドあるいは、ピロフェオフォルバイトは、F.C.Penningtonらの方法 (J.Am.Chem.Soc.86,1418(1964)) に従い、製造することができる。

例えば、Pyrophdはクロロフィルaをピリジンで処理することにより得られるピロクロロフィルを塩酸で処理して12位のフィチール基を除いて水素原子とし、またポルフィリン環のMg原子を水素原子にすることにより得ることができる。細胞内のクロロフィラーゼ活性や酸化酵素活性のない植物あるいはすでに単離されているクロロフィルを原料として化学的に製造する場合は、化学的に酸化及び脱フィチールすることを除けば、前述のクロロフィラーゼ及び酸化酵素活性のある植物を原料とする場合の製造法と同様の方法に従つて目的とする化合物を得ることができる。

この場合おだやかな酸化によつてクロロフィ

表-1から高活性OH-Phdと低活性OH-Phdは7,8位水素の立体配位の光学異性体と思われる。

本発明の制ガン剤における上記のクロロフィル誘導体の有効投与量はそのいずれもおよそ成人1日当たり10mg～300mg、好ましくは50～150mgである。

本発明の制ガン剤の製剤化にあたつては経口投与用製剤、あるいは注射用製剤のいずれでも通常行なわれる製剤化方法により製剤化が行われるが、注射用製剤とするにあたつてはPhd、OH-Phd共生理食塩水に直接溶解していくので、蒸留水に溶解した後、生理食塩水と混合して使用するのが良い。また、Phdはあらかじめ弱アルカリ性溶液に溶解した後中和し、生理食塩水と混和するのがよい。

次に上記物質の制ガン作用、毒性に関する薬理学的実験例及び本発明の制ガン剤の製造例、製剤化例をあげるが本発明はこれらの例示によつて肯定されるものではない。

実験例 1

ルからハイドロオキシクロロフィルを誘導した後蔗糖カラムクロマトグラフィーによりOH-Chldを分離し、後30%塩酸処理により脱フィチールすると、効率よくOH-Phdのみが得られる。

本発明者らは、高活性のOH-Phdと低活性のOH-Phdについて、調べた結果、以下の表-1に示す如きデータが得られた。

表-1

	高活性OH-Phd	低活性OH-Phd
分子式	C <sub>35</sub> H <sub>36</sub> O <sub>6</sub> N <sub>4</sub>	C <sub>35</sub> H <sub>36</sub> O <sub>6</sub> N <sub>4</sub>
E <sub>667</sub> /E <sub>409</sub>	1.93	1.99
R <sub>f</sub> (TLC)	0.34	0.21
ケミカルシフト (NMR)	8 4.73 7 4.47	4.45 4.09

注 E<sub>667</sub>/E<sub>409</sub> ; 可視部吸収スペクトルにおける青色極大吸収と赤色極大吸収の比

R<sub>f</sub>(TLC) ; シリカゲル薄層、20×20cm、0.25mm、展開溶媒、ベンゼン、エチルアセテート、エタノール、n-ブロパノール(14:4:1:1)でのR<sub>f</sub>値

ケミカルシフト 8} ; 核磁気共鳴における7、8位プロト  
7} ンのケミカルシフト

ザルコーマ180腫瘍細胞をICRマウス(雄7週令、約25g)の背部皮下にマウス1匹当たり1.25×10<sup>6</sup>個接種し、標準飼育し確実に腫瘍細胞の増殖を認めた個体(1群10匹)に移植後8日目から生理食塩水に溶かしたOH-Phdをマウス体重kg当たり0.10, 0.20mg又はPhd, Pyrophd, OH-Chld, Chld, Pyroehldを各々20mg/kg体重をマウスの腫瘍部位に直接投与した。試験期間中3日おきに9回の投与を行つた。直ちに波長400～1000nmの強度100mW/cm<sup>2</sup>の光(500Wタングステンランプの光を3cmの水層を通して熟練遮断)を1日、6時間照射した。腫瘍移植後32日目に腫瘍を摘出し、その重量を測定し次式によつて腫瘍抑制率を算出した。

$$\text{抑制率\%} = \frac{\text{対照区平均腫瘍重量} - \text{試験区平均腫瘍重量}}{\text{対照区平均腫瘍重量}} \times 100$$

対照群マウスは生理的食塩水を試験区と同様、腫瘍部位に投与し同様に光照射した。又OH-Phd 20mg/kg体重投与群を暗所で飼育し、暗所对照群とした。結果は表1に示されている。

Child, OH-Child, Pyrochild は不安定な物質で生体内中或いは抽出操作中分子中のMg原子が容易にはずれて、各々 Phd, OH-Phd, Pyrophd に変化する。

それらの抗腫瘍活性は各々対応する Mg-欠 Phd 細のそれとはほぼ同様であつた。

表1.クロロフィル誘導体の腫瘍直接投与による抗腫瘍効果

	総投与量 mg/kg	光	平均腫瘍 重量(g)		抑制率 (%)
			0	1.49±1.46	
対照区	0	L	1.49±1.46	0	
OH-phd	90	L	0.35±0.41	76.5	
	180	L	0.19±0.14	87.2	
	180	L*	0.75±1.04	49.7	
	180	D	1.43±0.86	4.0	
OH-Child	180	L	0.23±0.38	84.6	
Phd	180	L	0.63±0.32	57.7	
Child	180	L	0.54±0.47	63.8	
Pyrophd	180	L	0.86±0.72	42.3	
Pyrochild	180	L	0.57±0.42	61.7	

註 L; 光 20 Klux

L\*; 光 0.5 Klux

D; 暗

## 実験例 2

OH-Phd, Phd, 共に静脈投与は微量の投与で著効を示した。 OH-Phd 3.3 mg 投与照射群の中、約半数のマウスの腫瘍は消失した。

## 実験例 3

ザルコマ 180 を背部皮下に移植した ICR マウスを 23 日間標準飼育し移植腫瘍が約 200 ~ 300 mm<sup>2</sup> の大きさに増殖したマウスに OH-Phd 3 mg/kg 体重を尾静脈より投与、24 時間後に熱線を遮断した光強度 300 mW/cm<sup>2</sup> の光(光源熱線を遮断した光強度 300 mW/cm<sup>2</sup> の光(光源 500W, タングステンランプ)を 30 分間腫瘍部位へ照射した。 OH-Phd 投与、光照射処置は隔日に 3 回行ない、その後暗所飼育を続け、腫瘍の大きさの変化を観察した。 OH-Phd 無投与区は同量の生理食塩水を投与し、同様の光照射を行つた。

表3 OH-Phd の腫瘍治療効果

	OH-Phd 総投与量 (mg/kg 体重)	投与時腫瘍の 大きさ(mm <sup>2</sup> )	処置 10 日後の腫 瘍の大きさ(mm <sup>2</sup> )	抑制率 (%)	
				対照区	試験区
	0	273±64	376±123	0	
	9	248±82	129±79	66.0	

実験例 1 と同様に ICR マウス背部皮下にザルコマ 180 腫瘍細胞を移植後、標準飼育し、確実に腫瘍細胞の増殖を認めた個体に移植 8 日後から生理食塩水に溶かした OH-Phd 及び Phd をマウス体重 kg 当り 0 mg, 0.3 mg, 1 mg, 3 mg をマウス尾静脈より投与、2 ~ 3 日間隔で計 11 回投与し、実験例 1 と同様に光照射し 32 日間飼育後、腫瘍を摘出、腫瘍抑制率を調べた。

表2. OH-Phd の尾静脈投与による抗腫瘍効果

	総投与量 mg/kg 体重	光	平均腫瘍 重量(g)		抑制率 (%)
			0	5.54±1.04	
OH-Phd	3.3	L	1.05±0.6	81.0	
	1.1	L	0.68±0.60	87.7	
	3.3	L	0.18±0.18	96.8	
	3.3	L*	2.36±1.44	57.4	
	3.3	D	4.43±0.87	20.0	
Phd	3.3	L	1.6±0.87	71.1	
	1.1	L	1.3±1.44	76.3	
	3.3	L	2.3±1.29	57.9	

註 L; 20 Klux

L\*; 0.5 Klux

D; 暗

註 腫瘍の大きさ (mm<sup>2</sup>) ; 長径 × 短径 OH-Phd を投与し、光照射した区は腫瘍組織の変性壞死を生じ、処置 10 日後に処置前の約 1/3 の大きさ(体積換算)に縮少した。

## 実験例 4

実験例 1 と同様にザルコマ 180 を ICR マウスに移植し、移植後 8 日目から水に溶けた OH-Phd を 0.10 mg/kg 体重、胃ゾンデを用いて経口的にマウスに投与し、投与後、24 時間後 30 分間腫瘍部位に実験例 3 と同様 300 mW/cm<sup>2</sup> の光を照射した。投与および光照射処置は 5 日連続 2 回計 10 回行つた。32 日間飼育した後腫瘍を摘出し、その重量を測定して抑制率を算出した。

表4. OH-Phd の経口投与による抗腫瘍効果

	総投与量 (mg/kg)	平均腫瘍重量 (g)		抑制率 (%)
		対照区	OH-Phd 区	
	0	2.6±1.5	0.9±0.5	65.4
	1.00			

## 実験例 5 (毒性試験)

体重30g前後のICRマウス(雌、雄)を用いて、各投与経路による急性毒性試験を行なつた。

経口投与は蒸留水に溶解したものを胃ゾンデを用いて投与し、静脈内投与、腹腔内投与は生理食塩水に各々溶解し、注射器によつて行つた。LD<sub>50</sub>はリツチフィールド・ウイルコクソン法により算出した。投与後いずれも暗所で飼育した。

表5. 暗所における急性毒性 (LD<sub>50</sub>) mg/kg

投与	OH-Phd	Phd	Pyrophd
静脈内	200<	200<	200<
腹腔内	200<	200<	200<
経口	1000<	1000<	1000<

表中の数字は投与物質の水、生理食塩水への溶解度の限界を示すものであるが、いずれも死亡しなかつた。

これらの物質の正常細胞、臓器への吸收排泄は速やかで投与後12時間以内の光照射で光過敏症を呈するが、投与24時間以後の照射では何らの反応を示さなかつた。投与24時間後に

は各細胞、臓器に投与物質は殆ど認められなかつた。

#### 製造例 1

クロレラ細胞(湿体1kg)をリン酸緩衝液(0.1M, pH7.0)5Lに懸濁し、40℃で通気搅拌処理を48時間行なつた後、遠心分離を行ないこのクロレラ細胞を集めて、これに30%のアセトン溶液3Lを加えて36℃にて3時間静置する。

静置後、遠心分離を行ない上清を採取し、更に残渣に3Lのメタノールを3回加えて抽出液を得た。

得られた上清とメタノール抽出液の混液を減圧下に分離まで濃縮し、これにクロロホルムを2L加えて激しく混合後、蒸留水を加えて水洗を行ない、この後クロロホルム層を分離して、減圧下にクロロホルムを除去し残渣を得る。得られた残渣をエチルエーテル1Lに溶解し、等量の17%HCl溶液を加えた後、塩酸溶液層を分離し、水で5%の塩酸濃度まで希釈した後、

エチルエーテルを加えて混合する。このエーテル層を分離し水で洗浄した後、減圧下で濃縮する。この濃縮液をシリカゲル薄層に塗布して、溶媒(ベンゼン、エチルアセテート、エタノール、クロロプロパン、16:4:1:1)で冷暗所にて展開して色素バンド(R<sub>f</sub> 0.39とR<sub>f</sub> 0.34)を得た。

得られた色素バンドをかき取り、メタノールを加えて色素を抽出し、減圧下でメタノールを留去し、色素(Phd 1.19g, OH-Phd 0.86g)を得た。

#### 製造例 2

クロレラ生細胞濃縮液にアセトンを30%濃度に加え、pH7.0温度36℃で激しく通気24時間後、製造例1に従いクロロフィル系色素を抽出分離精製した。

クロレラ藻体100gからPhd 4.92mg, OH-Phd 3.86mgが得られた。

#### 製造例 3

クロレラ乾燥粉末(クロロフィラーゼ不活性)

をホモジナイザーで細胞破碎後30%アセトン溶液に懸濁し、24時間通気搅拌後、製造例1に従つてクロロフィル系色素を抽出し、次いで蔗糖カラムクロマトグラフィー(展開溶媒0.5%イソプロパノール・石油エーテル)でハイドロオキシクロロフィル画分を分離し、溶媒留去後エチルエーテルに溶解し、等量の30%塩酸を加え暗所下、室温で1時間振盪して脱フィチール化を行つた後、水を加えて塩酸濃度を17%とし、エーテルを加えて色素を分配、17%HCl画分をとり、以下製造例1と同様に精製した。クロレラ藻体100gからOH-Phd 6.18mgが得られた。

#### 製造例 4

精製したクロロフィル<sup>(50%)</sup>を100mgをアセトンに溶かし、シリカゲル(珪酸ソーダ)60gを加えてクロロフィルを吸着し、アセトンを揮発させた後、暗所下空気中で36℃に1時間放置する。該吸着した色素をアセトンで溶出し、暗所低温下で減圧濃縮し、エチルエーテルに溶か

し以下製造例3と同様にして蔗糖カラムクロマトグラフィーにかけて、ハイドロオキシクロロフィル画分をとり、エーテルに溶解し、等量の30% HCl溶液を加え、脱フィチール後、水を加えて17% 塩酸濃度とし以下製造例1と同様処理後精製した。

クロロフィル100mgからOH-Phd 32mgが得られた。

#### 製剤化例 1

OH-Phd 1.5mgを滅菌蒸留水0.5mlに溶解した後、1.8% 食塩水0.5mlで希釈後、除菌フィルターで沪過して、無菌的に注射用アンプルに充てんし、暗所に保存した。

#### 製剤化例 2

OH-Phd 630mg、Phd 870mgの混合物1500mgを0.1N NaOH溶液50mlに溶解後0.1N HCl溶液約50mlを加えて中和する。更に2% 食塩水を加えて150mlとする。次いで除菌フィルターで沪過して、無菌的に注射用アンプルに充てん、栓閉し、暗所に保存した。